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Intramolecular Masking of Nuclear Import Signal on NF-AT4 by Casein Kinase I and MEKK1

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Summary

T cell activation requires the import of NF-AT transcription factors to the nucleus, a process promoted by calcineurin-dependent dephosphorylation and inhibited by poorly understood protein kinases. Here, we report the identification of two protein kinases that oppose NF-AT4 nuclear import. Casein kinase Iα directly binds and phosphorylates NF-AT4, resulting in the inhibiton of NF-AT4 nuclear translocation. MEKK1 indirectly suppresses NF-AT4 nuclear import by stabilizing the interaction between NF-AT4 and CKIα. CKIα thus acts to establish an intramolecular masking of the nuclear location signal on NF-AT4, while MEKK1 augments this mechanism, and may further provide a link to signal transduction pathways regulating NF-AT4.

Introduction

The Rel transcription factors, including NF-κB (p50, p65, p105), c-Rel, and Rel-B, reside in the cytoplasm of cells and translocate to nucleus upon stimulation by cytokines or various environmental stresses (Baeuerle and Baltimore, 1996). NF-κB is restricted to the cytoplasm in unstimulated cells by its association with IκB, which binds to and masks the nuclear location signal (NLS) on NF-κB. Consequently, a common effect of the numerous signals that activate NF-κB is the destruction of IκB molecules. Recent experiments have defined the pathways of IκB degradation involving phosphorylation on Ser-32 and Ser-36 by a cascade of cytokine-activated kinases, followed by ubiquitin-directed proteolysis (Woronicz et al., 1997; Zandi et al., 1997).

A separate example of conditional nuclear import is displayed by the NF-AT transcription factors (NF-AT1/p, NF-ATc, NF-AT3, and NF-AT4), which are required for T cell activation (Rao et al., 1997). NF-ATs are cytoplasmic in unstimulated cells but, upon engagement of the T cell receptor (TCR) and CD28 coreceptor, rapidly translocate into the nucleus, where they activate an array of cytokine genes. Despite these parallels, the mechanism controlling NF-AT nuclear import is distinct from that regulating NF-κB/Rel activity. Nuclear import of NF-ATs is induced by the calcium-dependent phosphatase calcineurin (Jain et al., 1993; Loh et al., 1996; Shibasaki et al., 1996; Beals et al., 1997a; Masuda et al., 1997).

Another major difference between the nuclear import of NF-ATs and that of the NF-κB/Rel transcription factors concerns the reversability of the process. For instance, once nuclear import has been triggered by the degradation of IκB, the NF-κB/Rel proteins remain in the nucleus until new IκB molecules are synthesized, a process that may take several hours (Baeuerle and Baltimore, 1996). In contrast, the nuclear localization of NF-ATs is dependent upon continued calcium signaling, the cessation of which results in a rapid rephosphorylation and export of NF-AT to the cytoplasm in a matter of minutes (Shibasaki et al., 1996). In addition, the recycling of NF-AT occurs in the absence of de novo protein synthesis, while the cytoplasmic sequestration of activated NF-κB requires the synthesis of new IκB molecules. The rapid recycling of NF-AT is consistent with a molecular control mechanism involving phosphorylation-dependent, intramolecular masking of nuclear localization signals on NF-AT. The C terminus of NF-AT encompasses the Rel-like DNA-binding domain but is dispensable for calcium-dependent nuclear shuttling. In contrast, the N-terminal portion is the primary site of NF-AT phosphorylation and is sufficient for nuclear import-export trafficking of NF-AT (Shibasaki et al., 1996). Although GSK-3 appears to indirectly promote the nuclear export of NF-ATc (Beals et al., 1997b), significantly less is known about NF-AT kinases that directly oppose calcineurin.

In this study, we describe the identification of two protein kinases, one of which directly phosphorylates NF-AT4, and their respective and synergistic roles in suppressing NF-AT4 nuclear import.

Results

NF-AT4 Domains Required for Nuclear Import Dynamics

The N-terminal region of NF-AT4, NF-AT4(N) (aa 1-351) confers calcium-dependent nuclear import dynamics to green fluorescent protein (GFP) (Figure 1A and 1B). The N terminus of NF-AT4 is also the site of phosphorylation, as well as the site of binding and dephosphorylation by calcineurin (Shibasaki et al., 1996; Wesselborg et al., 1996; Rao et al., 1997). We examined the kinetics of calcium-dependent nuclear import and export of GFP-NF-AT4 and GFP-NF-AT4(N) in BHK cells. NF-AT4(N) and NF-AT4 showed identical nuclear shuttling dynamics in BHK cells, with a $t_{1/2}$ of 5 and 12 min for nuclear import and export, respectively (Figure 1B, and data not shown).

To define the subdomains within NF-AT4(N) critical for nuclear shuttling, we assayed a series of deletion

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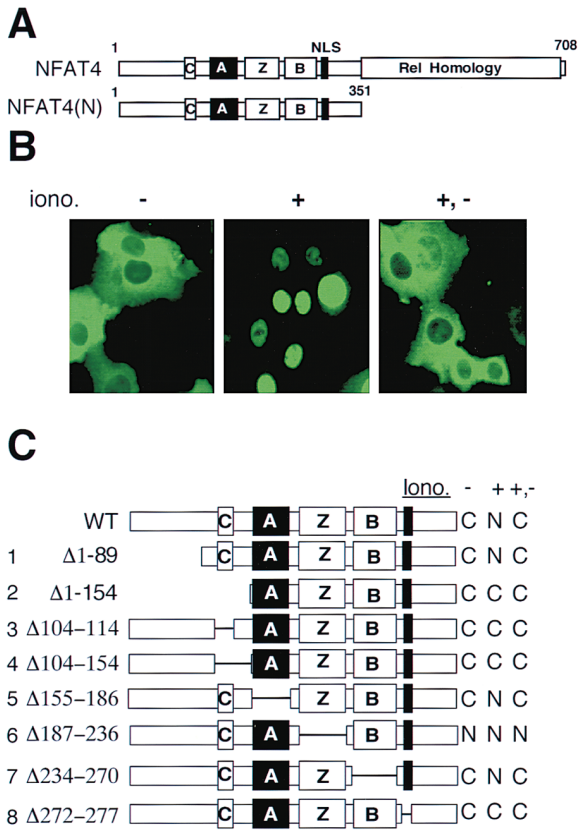

Figure 1. NF-AT4 Domains Required for Nuclear Shuttling Dynamics

(A) Schematic representation of NF-AT4, including a C-terminal Rel-homology (DNA binding) domain and the N-terminal regulatory domain controlling calcium-dependent nuclear shuttling; C, putative calcineurin binding site; A, serine rich domain; Z, putative NLS masking domain; B, a possible linker region; NLS, putative nuclear location signal.

(B) Nuclear shuttling of GFP-NF-AT4(N) in BHK cells. (−), unstimulated; (+), treated with A23187 (1 μM) for 30 min; and (+,−), treated with calcium ionophore for 30 min followed by 30 min wash.

(C) Analysis of shuttling dynamics of NF-AT4(N) mutants in resting (−), calcium activated (+), and postactivation (+,−) BHK cells. C, predominantly cytoplasmic localization; N, predominantly nuclear localization.

mutants (Figure 1C). Loss of the extreme N terminus had no effect on calcium-dependent NF-AT4 dynamics (Figure 1C, mutant 1). However, further deletion into the N-terminal domain marked by a NF-AT consensus sequence, GxxxxxxPxIxIT (aa 102-114, C domain), resulted in a constitutively cytoplasmic localization of NF-AT4(N) (Figure 1C, mutants 2, 3, and 4). This sequence is within a region recently shown to be necessary for calcineurin binding (Loh et al., 1996; Wesselborg et al., 1996; Masuda et al., 1997). Significantly, coexpression of calcineurin (subunits CnA and CnB) resulted in the rapid, calcium-dependent translocation of the NF-AT4(N)-ΔC mutant to the nucleus (data not shown), supporting the notion that the GxxxxxxPxIxIT sequence functions to enhance the local concentration of calcineurin on NF-AT4.

The A and B domains of NF-AT4 were defined as regions conserved in all NF-AT isotypes (Hoey et al., 1995). However, removal of either the A or the B domain had no obvious effect on the nuclear shuttling of NF-AT4(N) (Figure 1C, mutants 5 and 7). In contrast, removal of the intervening Z domain (aa 187-236; mutant 6) rendered NF-AT4(N) constitutively nuclear, even in the absence of calcium ionophore. The Z domain, therefore, appears to be essential for maintaining NF-AT4(N) in the cytoplasm, possibly through masking of an NLS sequence. The location of the NLS sequence within NF-AT4(N) appears to be the basic region at aa 272-277, which shows homology to consensus NLSs (Gorlich, 1997). The deletion of this putative NLS blocked the nuclear import of NF-AT4(N) (Figure 1C, mutant 8). In contrast to the ΔC mutant, the nuclear import defect of mutant 8 was not rescued by overexpressed calcineurin (data not shown), suggesting that aa 272-277 acts as a critical nuclear location signal. Taken together with the concept of intramolecular masking (Shibasaki et al., 1996; Beals et al., 1997), three functional elements are required for the nuclear shuttling of NF-AT4(N): a calcineurin binding site (C domain), a nuclear location signal, and a putative NLS masking domain (Z domain).

Affinity Purification of NF-AT4 Kinases

Pilot experiments showed that NF-AT4 kinase activity in HeLa cell lysates was depleted by preincubation with GST-NF-AT4(N), but not with GST (data not shown), indicating the feasibility of an affinity purification approach. Large-scale affinity purification was then performed using ammonium sulfate fractions of HeLa cell lysates preabsorbed with GST and applied to a column of GST-NF-AT4(N). After extensive washing, bound proteins were eluted with a NaCl step gradient and assayed for NF-AT4 kinase activity. This analysis revealed a peak of NF-AT4 kinase activity that eluted at 0.5 M NaCl. Mono-Q ion exchange chromatography of the eluted proteins resolved a single peak of NF-AT kinase activity, corresponding to a single protein species with an apparent molecular weight of 40 kDa (Figure 2A). Tryptic peptides from this 40 kDa species were fractionated by HPLC and analyzed by mass spectrometer and Edman degradation sequencing. The derived peptide sequences were identical to those of casein kinase Iα (CKIα) (Rowles et al., 1991).

CKIα: An NF-AT Kinase

We next examined whether the overexpression of CKIα would affect calcium-dependent NF-AT4 nuclear import. GFP-NF-AT4(N) was expressed either alone or together with HA-tagged CKIα in BHK cells. The cells were then treated with 1 μM calcium ionophore (A23187) for different periods of time. Nuclear import of GFP-NF-AT4(N) was complete within 10 min of calcium ionophore treatment (Figures 2B and 2C). In contrast, cells coexpressing CKIα showed very inefficient nuclear import of GFP-NF-AT4(N) at the same time point, with over 95% of cells displaying predominantly cytoplasmic GFP-NF-AT4(N) (Figures 2B and 2C). After 30 min of calcium ionophore treatment, however, approximately 50% of CKIα-expressing cells showed predominantly nuclear NF-AT4 (Figures 2B and 2C), and by 50 min almost 80%

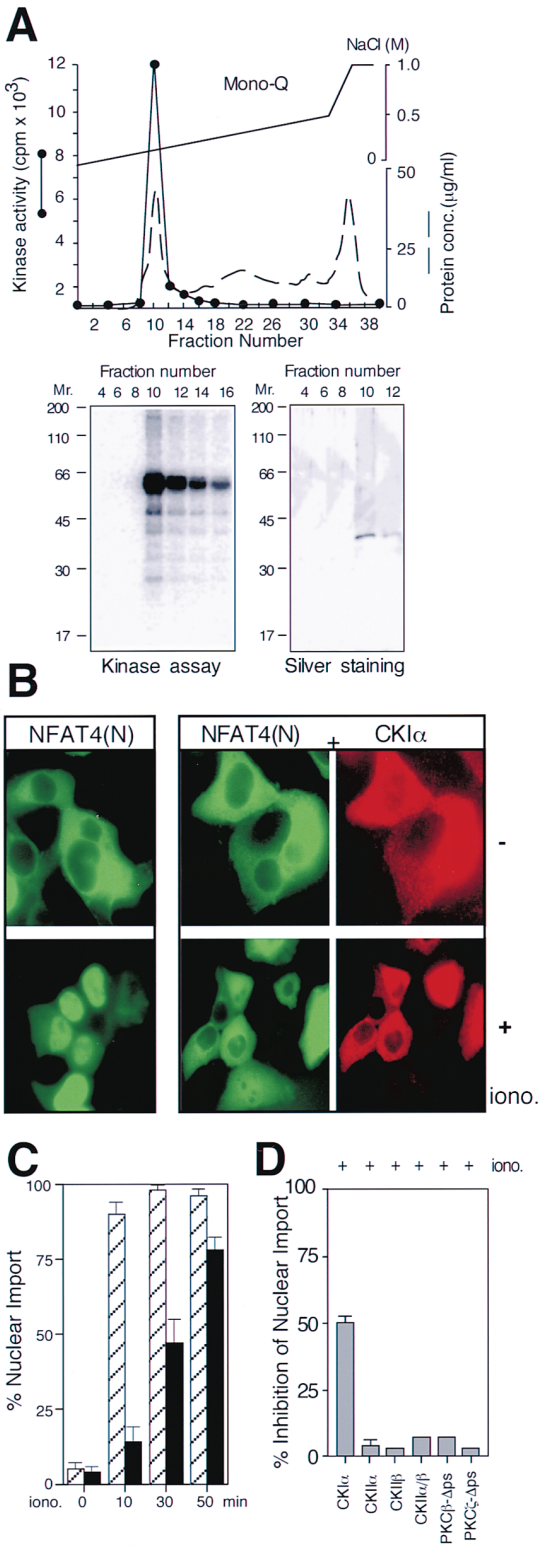

Figure 2. Identification of CKIα as NF-AT4 Kinase

(A) Mono Q ion-exchange chromatography profile of proteins eluted from GST-NF-AT4(N) affinity column. Fractions were assayed for NF-AT4 kinase activity using GST-NF-AT4(N) as substrate. Fractions containing kinase activity were resolved by SDS-polyacrylamide gel electrophoresis and silver stained to reveal a protein of approximately 40 kDa.

showed nuclear NF-AT4 (Figure 2C). It appeared, then, that prolonged activation of calcineurin compensated for the effect of elevated CKIα activity. As controls, we tested casein kinase II (CKII α and β subunits), protein kinase C beta (PKCβ), and protein kinase C zeta (PKCζ). Neither CKII, PKCβ, nor PKCζ showed inhibition of NF-AT4 nuclear import (Figure 2D).

MEKK1 Suppresses NF-AT4 Nuclear Import

In a parallel strategy of identifying NF-AT4 kinases, we observed that NF-AT4(N) responds very poorly to calcium ionophores when expressed in several human cell lines (Shibasaki et al., 1996). In U2OS cells, less than 10% of transfected cells showed nuclear NF-AT4(N) after 30 min of calcium ionophore treatment, whereas BHK cells typically show a 95% response at this time point. To test the notion that this refractory state was dependent on growth factor signaling pathways, U2OS cells expressing NF-AT4(N) were serum-starved for different periods of time and then treated with calcium ionophore. Significantly, serum deprivation markedly enhanced the ability of NF-AT4 to undergo calcium-dependent nuclear import, suggesting that NF-AT4 translocation is inhibited by serum-responsive kinases (Figure 3A).

Using a candidate kinase approach, we screened an array of growth factor-responsive kinases for ones that suppress NF-AT4(N) nuclear import. NF-AT4(N) was coexpressed with either p90RSK, p70S6K, p38Hog1, MEKK1, JNK1, SEK1, ERK1 (constitutively active mutant), or MKK3 (constitutively active mutant) in BHK cells and assayed for nuclear import in response to calcium ionophore. While most of these kinases did not affect NF-AT4 dynamics, MEKK1 displayed a remarkable, inhibitory effect on NF-AT4 nuclear import (Figure 3B).

Serum Deprivation Inactivates MEKK1

We next asked whether serum deprivation affects endogenous MEKK1 activity in U2OS cells. MEKK1 is activated by a proteolytic event that removes its large, N-terminal inhibitory domain (Cardone et al., 1997). To determine whether serum deprivation affects MEKK1 activity in U2OS cells, we used a C-terminal–specific MEKK1 antibody in Western blots to determine the ratio between the full-length, inactive MEKK1 (~200 kDa) and the active proteolytic fragment (~80 kDa) (Cardone et al., 1997). In the presence of serum, MEKK1 was found to exist almost exclusively as the active, 80 kDa species (Figure 3C). However, upon 1 hr of serum starvation, MEKK1 appeared as the inactive, 200 kDa species. The

(B) Immunofluorescence staining of BHK cells expressing GFP-NF-AT4(N) alone (left) or with HA-CKIα (middle, right) in the absence (top) or presence (bottom) of calcium ionophore. Bottom panel shows corresponding protein localization after treatment with calcium ionophore for 30 min.

(C) Quantitation of NF-AT4(N) nuclear import in response to different times of ionophore exposure in cells expressing GFP-NF-AT4(N) alone (hatched) or together with HA-CKIα (solid).

(D) Effect of constitutively active kinases on NF-AT4(N) nuclear import in response to calcium ionophore. GFP-NF-AT4(N) was coexpressed with indicated kinases in BHK cells. The transfected cells were then treated with calcium ionophore for 30 min.

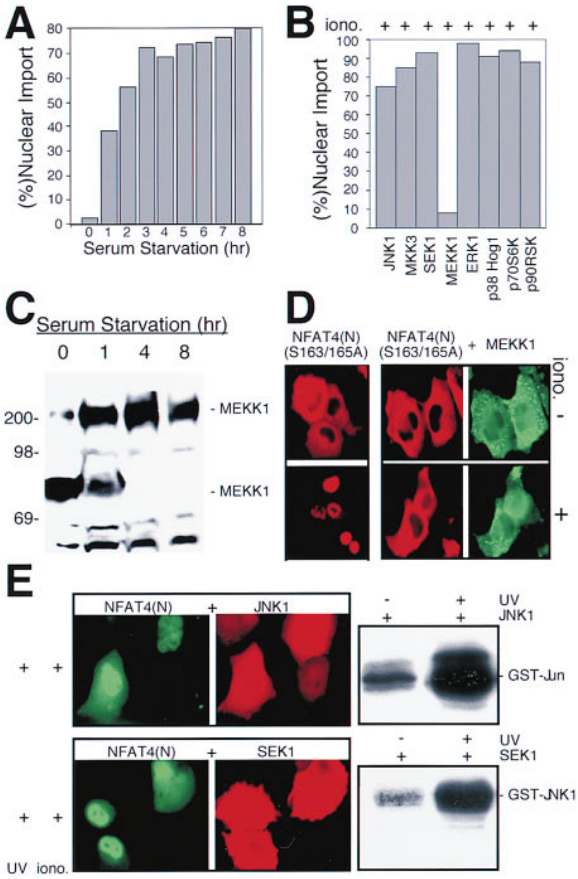

Figure 3. Inhibition of NF-AT4(N) Nuclear Import by MEKK1

(A) Effect of serum starvation on calcium ionophore-stimulated nuclear import of GFP-NF-AT4(N) in U2OS cells. U2OS cells expressing GFP-NF-AT4(N) were serum-deprived for the indicated times, treated with ionophore for 30 min, and analyzed for nuclear import of GFP-NF-AT4(N).

(B) Nuclear import assay of GFP-NF-AT4(N) in cells coexpressing indicated kinases in BHK cells treated with ionophore for 30 min.

(C) Inactivation of endogenous MEKK1in U2OS cells following intervals of serum starvation. Endogenous MEKK1 was immunoprecipitated with a C-terminal–specific antibody and detected by immunoblotting with the same antibody.

(D) Inhibition of NF-AT4(N)(S163/165A) nuclear translocation by MEKK1. NF-AT4(N) lacking JNK consensus phopsphorylation sites S163 and S165 is cytoplasmic in resting cells and responds by nuclear translocation to ionophore stimulation (left). Coexpression of MEKK1 does not prevent the nuclear import of NF-AT4(N)(S163/165A) upon ionophore stimulation (middle, right panels).

(E) JNK and SEK1 fail to block GFP-NF-AT4(N) nuclear import, even when hyperactivated by UV irradiation. GFP-NF-AT4(N) was transiently expressed with SEK1 (top panel) or JNK1 (bottom panel) in BHK cells, which were sequentually treated with UV radiation and calciom ionophore. GFP-NF-AT4(N) (left panel) was imported to the nucleus, despite the hyperactivation of SEK1 and JNK1 indicated by biochemical analysis (right panel). The increases in JNK1 and SEK1 activities after UV irradiation were monitored by in vitro kinase assays using GST-Jun(1-79) or GST-JNK1(K/R) as substrate, respectively.

proteolytic activation of MEKK1 was completely blocked as early as 4 hr of serum starvation (Figure 3C). The kinetics of MEKK1 inactivation, therefore, appear to parallel those of NF-AT4 nuclear translocation under serum-starvation conditions (Figures 3A and 3C).

MEKK1 Does Not Act through JNK to Suppress NF-AT Nuclear Import

MEKK1 activates stress signal pathways through its direct phosphorylation SEK/MKKs, which in turn activates JNKs and p38 (Sanchez et al., 1994; Yan et al., 1994). However, our experiments with BHK cells suggested that SEK1, JNK1, and p38/Hog1 had no effect on the ability of NF-AT4 to translocate to the nucleus (Figure 3B). It was recently reported that JNK1/2 phosphorylate Ser-163 and Ser-165 on NF-AT4 (Chow et al., 1997). To determine whether the ability of MEKK1 to suppress NF-AT4 nuclear import is mediated by the stress pathway involving SEK1 and JNK, we tested NF-AT4(N), mutated at the JNK phosphorylation sites, in our system. Significantly, the calcium-dependent nuclear shuttling of the NF-AT4(N)(S163/165A) mutant was indistinguishable from wild-type NF-AT4 (Figure 3D). MEKK1 completely blocked the calcium-dependent nuclear translocation of NF-AT4(N)(S163/165A) (Figure 3D). The nuclear import of full-length NF-AT4(S163/165A) was also blocked by MEKK1 (data not shown).

To further confirm that the activation of SEK/JNK does not impair NF-AT4 nuclear translocation, UV irradiation was used to hyperactivate GST-tagged JNK1 or SEK1 (Sanchez et al., 1994). JNK1 and SEK1 activities were monitored by in vitro kinase assays using either GST-Jun or GST-JNK1(K/R) as substrates, respectively (Figure 3E). Despite the hyperactivation of JNK1 or SEK1, neither had an effect on NF-AT4(N) nuclear translocation (Figure 3E).

Dominant-Negative CKIα Induces Nuclear Import of NF-AT4

To address the mechanism underlying the suppression of NF-AT4 nuclear import by CKIα, we generated a dominant-negative CKI (D136N) that retains less than 10% wild-type kinase activity (Figure 4A). Significantly, when NF-AT4(N) was coexpressed with CKIα(D136N), about 40% of cotransfected cells showed predominantly nuclear NF-AT4(N) in the absence of calcium ionophore treatment (Figures 4B and 4C). Upon stimulation with calcium ionophore, GFP-NF-AT4(N) translocated to the nucleus in the remaining cells. Curiously, the cells coexpressing CKIα(D136N) showed a marked retardation in NF-AT4(N) nuclear export after the withdrawl of calcium ionophore (Figures 4B and 4C). We further noted that CKIα(D136N) colocalized with GFP-NF-AT4(N) in the nucleus, suggesting their association and cotransport (Figure 4C).

Given that dominant-negative CKIα promoted NF-AT4(N) nuclear import, we asked which regions of NF-AT4(N) were required for this effect. CKIα(D136N) was coexpressed with NF-AT4(N) deletion mutants in BHK cells (Figure 4D). NF-AT4(N)ΔC, which lacks a calcineurin binding site, failed to translocate to the nucleus in response to calcium ionophore (Figure 1C). However, coexpression with CKIα(D136N) restored the NF-AT4(N)ΔC nuclear import response (Figure 4D), indicating that dominant-negative CKIα blocks the mechanism of cytoplasmic sequestration of NF-AT4, and thereby functionally enhances calcineurin activity toward NF-AT4. We then tested whether the A domain was required

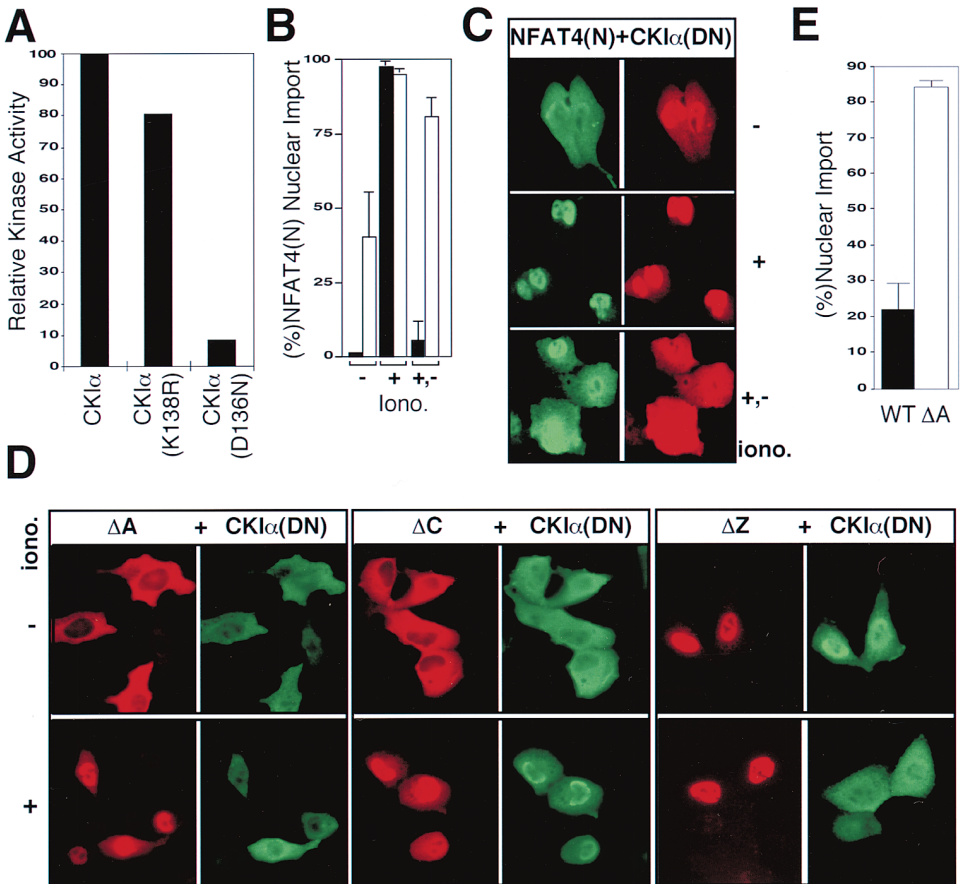

Figure 4. Dominant-Negative CKIα Induces NF-AT4(N) Nuclear Import

(A) Relative kinase activity of CKIα catalytic domain mutants immunoprecipitated from BHK cells and assayed against CKI-specific peptide substrate. Kinase activity is expressed relative to that of wild-type CKIα.

(B) GFP-NF-AT4(N) was expressed with (open) or without (solid) HA-CKIα(D136N) in BHK cells. Nuclear import of NF-AT4 was determined in the absence (−) or presence of ionophore for 30 min (+), or after treatment with ionophore for 30 min followed by a 60 min washout (+,−).

(C) Representative immunofluorescence images for (B).

(D) Effect of dominant-negative CKIα(D136N) on nuclear import of NF-AT4(N) mutants. Myc-tagged NF-AT4(N)-ΔA, -ΔC, and -ΔZ mutants were individually coexpressed with HA-CKIα (D136N) in BHK cells. Cells were either left untreated (−) or treated with ionophore for 30 min (+).

(E) NF-AT4(N) ΔA mutant is hypersensitive to calcineurin activation. BHK cells expressing either the wild-type and the ΔA mutant were treated with a low concentration (200 nM) of calcium ionophore for 10 min. The percentage of transfected cells showing predominantly nuclear NF-AT4 nuclear localization is indicated.

for the effect of dominant-negative CKIα. CKIα(D136N) failed to induce nuclear import of NF-AT4(N)ΔA in the absence of calcium ionophore. Calcium ionophore treatment caused NF-AT4(N)ΔA nuclear translocation, while CKIα(D136N) remained in cytoplasm (Figure 4D). This lack of colocalization supports the idea that the A domain of NF-AT4 is essential for CKIα/NF-AT4 interaction. Finally, coexpression of CKIα(D136N) with the constitutively nuclear NF-AT4(N)ΔZ mutant led to predominantly nuclear localization of CKIα(D136N), further suggesting a physical interaction between NF-AT4 and CKIα. However, CKIα(D136N) returned to cytoplasm after calcium ionophore treatment (Figure 4D). One interpretation of this result is that the association between CKIα and NF-AT4 is phosphorylation-dependent and disrupted by activated calcineurin.

Since the A domain appeared to mediate NF-AT4/CKIα association, we tested whether deletion of the A domain would alter the sensitivity of NF-AT4 toward calcineurin activation. BHK cells expressing either wild-type NF-AT4(N) or the ΔA mutant were treated with a low concentration (200 nM) of calcium ionophore for 10 min. Wild-type NF-AT4(N) was refractory to low concentrations of ionophore, with only 30% of cells showing nuclear import after 10 min. In contrast, the ΔA mutant was hypersensitive to calcium ionophore stimulation, with 90% of transfected cells responding with nuclear NF-AT4(N) (Figure 4E). Therefore, the A domain of NF-AT4 appears to promote the action of NF-AT kinases which oppose calcineurin.

In Vivo Association between NF-AT4 and CKIα

To test the notion that the A domain mediates the interaction with CKIα, HA-tagged CKIα(D136N) was coexpressed with Myc-tagged NF-AT4 deletion mutants in BHK cells. NF-AT4 mutants were immunoprecipitated with the anti-Myc antibody 9E10, and associated CKIα(D136N) was detected with an anti-HA antibody

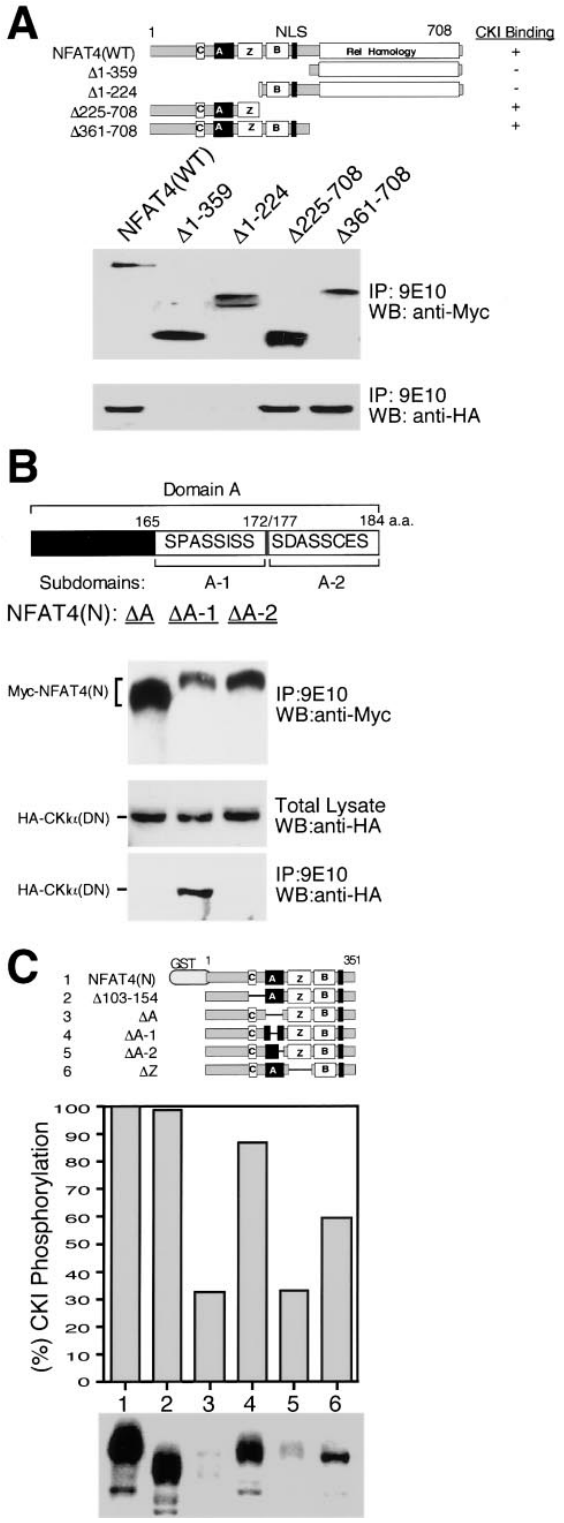

Figure 5. In Vivo NF-AT4/CKIα Association and In Vitro Phosphorylation of NF-AT4

(A) Myc-tagged NF-AT4 mutants were individually coexpressed with HA-tagged CKIα(D136N) in BHK cells, immunoprecipitated, and associated HA-CKIα(D136N) detected by Western blotting with an anti-HA-antibody.

(B) CKIα interacts with the A-2 subregion. Myc-tagged NF-AT4(N)-ΔA, ΔA-1, and ΔA-2 were individually coexpressed with HA-tagged CKIα(D136N). Coimunoprecipitation and immunoblotting were done as in (A).

(C) In vitro phosphorylation of GST-NF-AT4(N) deletion mutants by purified CKI. GST-NF-AT4(N) mutants were used as substrates for purified CKI catalytic fragment. The phosphorylation reaction proceeded briefly for 1 min at room temperature. ^{32}P-incorporation was quantitated by Phospho-imager and presented with the autoradiograph.

(Figure 5A). This analysis indicated that the N-terminal region of NF-AT4 was essential for CKIα binding (Figure 5A). The CKIα binding domain on NF-AT4 was further localized to the A domain, as the A domain deletion mutant showed no association with CKIα(D136N) (Figure 5B). The analysis of two subdeletions within the A domain, ΔA-1 (ΔSPASSISS, aa 165-172) and ΔA-2 (ΔSDASSCES, aa 177-184), revealed that the A-2 subregion within the A domain was essential for CKIα association (Figure 5B).

We next examined whether the A domain was required for efficient phosphorylation of NF-AT4(N) by CKIα. To do this, NF-AT4(N) deletion mutants were produced as GST fusion proteins and briefly phosphorylated by highly purified CKI (Figure 5C). Whereas high levels of phosphate incorporation were detected on GST-NF-AT4(N), mutants lacking the A domain or the A-2 subdomain were much less phosphorylated by CKI (Figure 5C). Noticeably, the deletion of the Z domain resulted in a 40% decrease in phosphorylation, suggesting that the Z domain also contains CKIα phosphorylation sites (Figure 5C). The A and Z domains were also individually expressed as GST fusion proteins, both of which proved to be excellent substrates of CKIα in vitro (data not shown).

Phosphorylation-Dependent Masking of Nuclear Location Signal

Having identified the regions of NF-AT4 phosphorylated by CKIα, we sought to map the CKI phosphorylation sites that are crucial for NLS masking. A 165–amino acid region of NF-AT4 (aa 187-351) containing the Z domain and the NLS was phosphorylated in vitro by CKI, digested with trypsin, and the resultant peptides analyzed by reverse phase HPLC C18 and direct sequencing. This analysis revealed a single, highly phosphorylated peptide (no. 39) having amino acids FTGSxLT at the N terminus and SLSPR at the C terminus, corresponding to aa 203-238 within the Z domain of NF-AT4 (Figure 6A). We estimated that approximately 5–6 phosphate groups were incorporated onto this peptide, primarily within the closely spaced S/T residues (TxxSxxTSxxxS, aa 204-215) at its amino terminus.

To evaluate the functional significance of CKI phosphorylation sites in the Z domain on NF-AT4 dynamics, we mutated the five S/T residues within aa 204-215 to alanine (Figure 6B). These mutations were first introduced into NF-AT4(N)(187-351). Wild-type NF-AT4(N) (187-351) localized to the cytoplasm, indicating that the regions upstream of the Z domain are not crucial for the NLS masking (Figure 6B). In contrast, the mutant NF-AT4(N)(187-351)(Ala) was predominantly nuclear (Figure

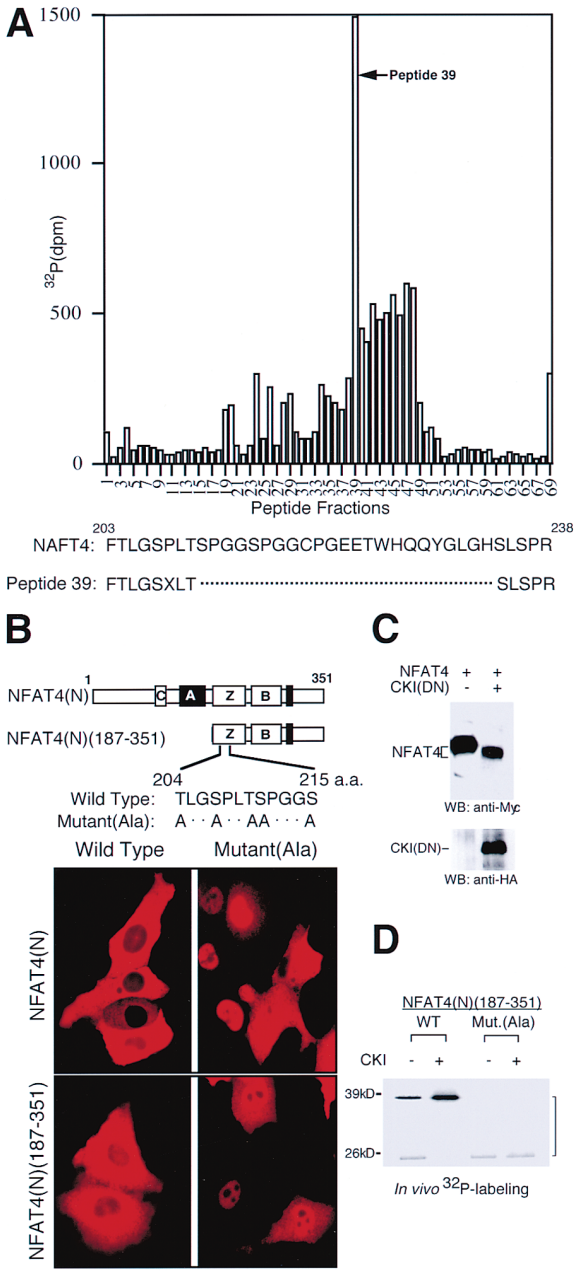

Figure 6. Identification of CKIα Phosphorylation Sites and Phosphorylation-Dependent NLS Masking of NF-AT4

(A) Identification of CKIα phosphorylation sites by phosphopeptide sequencing. Baterially expressed 6×His-tagged NF-AT4(187-351) was in vitro phosphorylated by CKI. Trypsin digested peptides were separated on HPLC reverse phase C18 column, and ^{32}P-incorporation on each peptide was counted. ^{32}P counts (dpm) were plotted against peptide fraction numbers. The N-terminal and C-terminal amino acids of peptide 39 determined by direct sequencing are aligned with the corresponding region of NF-AT4.

(B) Phosphorylation-dependent NLS masking of NF-AT4. The serine/threonine residues between aa 204-215 were mutated to alanine. The mutations were introduced into NF-AT4(N) and NF-AT4(N)(187-351). The wild-type and mutant NF-AT4 proteins were transiently expressed in BHK cells. The cellular localization was visualized by immunofluorescence.

(C) Dominant-negative CKIα blocks phosphorylation of NF-AT4 in vivo. Myc-tagged full-length NF-AT4 was expressed with or without dominant-negative CKIα in BHK cells. The transfected cells were treated with calcium ionophore for 30 min and then incubated in the absence of calcium ionophore for additional 30 min. NF-AT4 was detected by Western blotting with an anti-Myc antibody.

(D) In vivo phosphorylation of NF-AT4 by CKIα. Myc-tagged, wild-type NF-AT4(N)(187-351) or the mutant NF-AT4(N)(187-351)(Ala) was expressed with or without CKIα in BHK cells. Transfected cells were labeled with ^{32}P-phosphate. The amount of NF-AT4 protein was first examined by Western blotting an aliquot of cell lysate with anti-Myc antibody. Cell lysates containing similar amount of NF-AT4 proteins were then immunoprecipitated with anti-Myc mAb 9E10 and resolved on 13% SDS-PAGE gel. NF-AT4 proteins were detected by autoradiography.

6B). The same mutations were also introduced into NF-AT4(N), yielding a similar, predominantly nuclear, localization (Figure 7B). Significantly, single point mutations altering only one of these S/T residues did not apparently affect NF-AT4 cellular localization (data not shown).

We have demonstrated previously that NF-AT4 was rephosphorylated within 5 min after calcium ionophore withdrawal (Shibasaki et al., 1996). To determine whether CKIα affects the phosphorylation status of NF-AT4 in vivo, full-length NF-AT4 was transiently expressed in BHK cells, with or without CKIα(D136N). Transfected cells were treated with calcium ionophore for 30 min and then incubated in the absence of calcium ionophore for an additional 30 min. The phosphorylation of NF-AT4 was examined by observing changes in its electrophoretic mobility. Significantly, the coexpression of CKIα(D136N) substantially inhibited the rephosphorylation of NF-AT4 upon calcium ionophore withdrawal (Figure 6C).

Next, we asked whether CKIα specifically phosphorylates the S/T residues in the Z domain in vivo. To analyze the phosphorylation of these residues, we expressed NF-AT4(N)(187-351) or the mutant NF-AT4(N)(187-351)(Ala) with or without wild-type CKIα in BHK cells. The cells were then labeled with ^{32}P-phosphate. The majority of wild-type NF-AT4(N)(187-351) was hyperphosphorylated in vivo, as indicated by its slower mobility in SDS-PAGE gels. A minor fraction of this protein existed as a hypophosphorylated form with much higher mobility (Figure 6D). Coexpression of CKIα dramatically augmented the in vivo phosphorylation of NF-AT4(N)(187-351) and completely converted NF-AT4(N)(187-351) to the hyperphosphorylated form (Figure 6D). In contrast, the mutant NF-AT4(187-351)(N)(Ala) was minimally phosphorylated in vivo, and coexpression of CKIα did not increase its phosphorylation (Figure 6D). The in vivo phosphorylation was strikingly similar to the in vitro phosphorylation, in which bacterially expressed NF-AT4(N)(187-351) was converted, by CKI, into a hyperphosphorylated form with an apparent molecular weight of ~35 kDa (data not shown). These results demonstrated that the CKIα phosphorylation sites identified in vitro were also specifically phosphorylated by CKIα in vivo, and that these residues were crucial for the masking of the NLS of NF-AT4.

Suppression by MEKK1 Requires CKIα

Overexpression of MEKK1 efficiently suppressed the nuclear import of NF-AT4(N) (Figure 3C). An important question remains as whether MEKK1 acts on NF-AT4 independently or through CKIα. Given the requirement

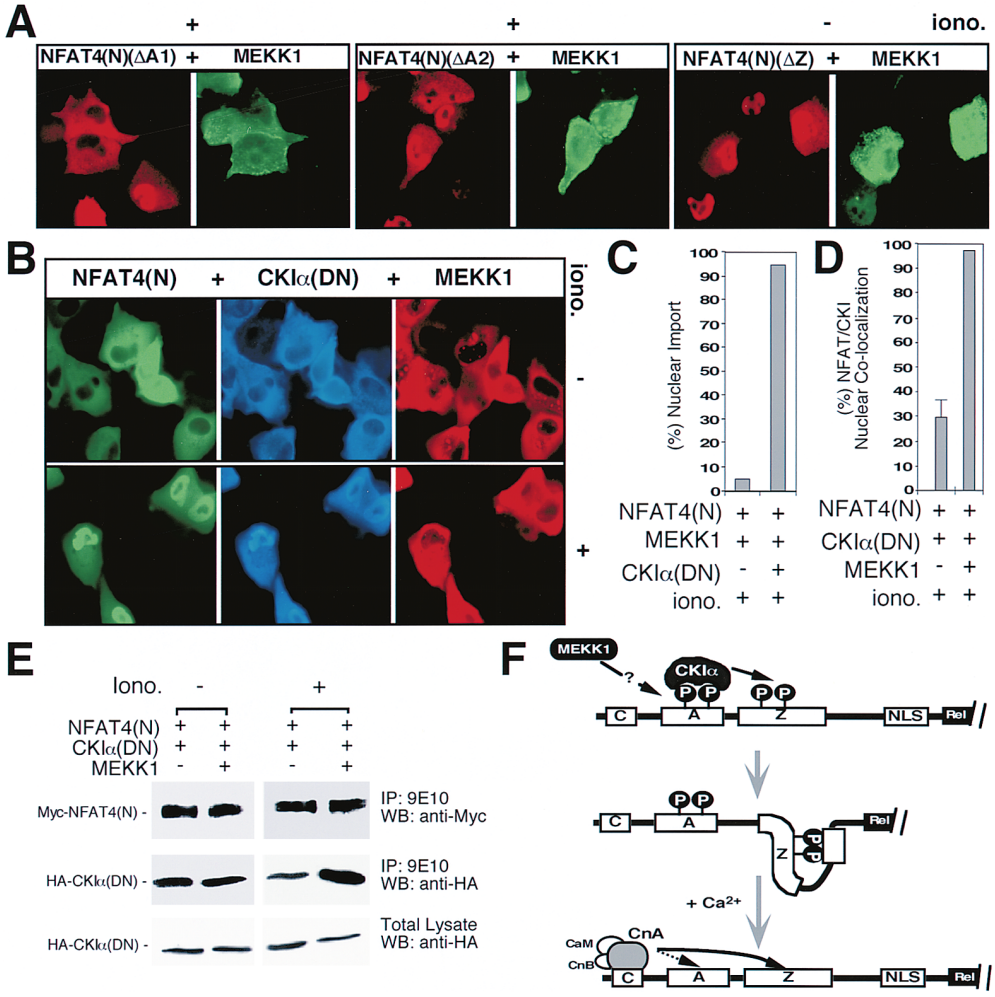

Figure 7. The Mechanisms of Suppression by MEKK1

(A) Suppression by MEKK1 requires the CKIα binding site of NF-AT4. HA-tagged MEKK1 was coexpressed with either Myc-tagged NF-AT4(N)ΔA-1, NF-AT4(N)ΔA-2, or NF-AT4(N)ΔZ in BHK cells. The cells were either untreated (−) (for NF-AT4(N)ΔZ) or treated with ionophore for 30 min (+), and then processed for immunofluorescence, as indicated.

(B) Suppression of NF-AT4(N) nuclear import by MEKK1 requires CKIα activity. GFP-NF-AT4(N), HA-CKIα(D136N), and GST-MEKK1 were coexpressed in BHK cells, which were then either untreated (−) or treated with ionophore for 30 min (+). Cells were then processed for immunofluorescense as indicated.

(C) Quantification of NF-AT4(N) nuclear import assay as in (B) as a function of CKIα(D136N).

(D) Percentage of cells showing nuclear colocalization of CKIα(D136N) and GFP-NF-AT4(N) as a function of MEKK1.

(E) MEKK1 enhances CKIα(D136N) binding to NF-AT4 in vivo. GST-MEKK1, Myc-NF-AT4(N), and HA-CKIα(D136N) were coexpressed in BHK cells. Cells were either left untreated (−) or treated with A23187 for 15 min (+). Myc-NF-AT4(N) was immunoprecipitated with anti-Myc antibody, and coprecipitated HA-CKIα(D136N) was detected by Western blotting with a polyclonal anti-HA antibody.

(F) Schematic model for the cooperation between CKIα and MEKK1 in suppressing NF-AT4 nuclear import.

of the A domain of NF-AT4 in mediating CKIα association, we tested whether MEKK1 could inhibit the nuclear import of the NF-AT4(N)ΔA1 and ΔA2 mutants. HA-MEKK1 and Myc-NF-AT4(N)ΔA-1 or ΔA-2 were coexpressed in BHK cells, which were subsequently stimulated with calcium ionophore (Figure 7A). MEKK1 completely blocked the nuclear translocation of NFAT4(N)ΔA-1 (Figure 7A) in response to calcium ionophore stimulation. In contrast, NF-AT4(N)ΔA-2, which lacks the CKIα binding site, rapidly entered the nucleus despite the presence of MEKK1 (Figure 7A), indicating that MEKK1 requires the CKIα binding site of NF-AT4 to exert its inhibitory effect. Furthermore, MEKK1 did not revert the constitutive nuclear localization of the NF-AT4(N)ΔZ mutant (Figure 7A).

To determine whether CKIα activity is required for MEKK1 to suppress NF-AT4 nuclear import, BHK cells were cotransfected with GFP-NF-AT4(N), HA-CKIα(D136N), and GST-MEKK1 and then stimulated with calcium ionophore (Figures 7B and 7C). In the presence of the dominant-negative CKIα, MEKK1 was unable to suppress the calcium-activated nuclear import of NF-AT4(N). Additionally, in the presence of MEKK1, the nuclear colocalization of CKIα (D136N) and NF-AT4(N) was dramatically enhanced, suggesting that MEKK1 augmented the association between NF-AT4(N) and CKIα(D136N) (Figure 7D).

MEKK1 Stabilizes the Interaction between CKIα and NF-AT4

Two observations have to be reconciled concerning the mechanism by which MEKK1 suppressed NF-AT4 nuclear import. First, CKIα binds the A domain and phosphorylates the domain Z, the region required for NLS masking. Second, inhibition by MEKK1 requires both the CKIα binding site in NF-AT4 and CKIα activity. These observations indicate that MEKK1 promotes the association between CKIα and NF-AT4. To test this notion, we examined whether MEKK1 affects the association between CKIα and NF-AT4(N). CKIα/NF-AT4(N) binding was assayed with lysates of BHK cells coexpressing Myc-tagged NF-AT4(N) and either HA-CKIα(D136N) or both HA-CKIα(D136N) and GST-MEKK1. In the absence of calcium ionophore stimulation, no apparent increase in CKIα(D136N)/NF-AT4(N) association was observed as a result of MEKK1 coexpression (Figure 7E). However, after 15 min of calcium ionophore treatment, MEKK1 coexpression enhanced the binding of CKIα(D136N) to NF-AT4(N) by nearly 10-fold (Figure 7E). MEKK1, therefore, strengthens the interaction between CKIα and NF-AT4(N) in the presence of activated calcineurin.

Discussion

Unique Nuclear Shuttling Mechanism of NF-AT4

In the present study, we focused on the Z domain of the N-terminal, regulatory region of NF-AT4, as it exhibited all the features expected for a NLS masking element. For one, it is the only region of NF-AT4 whose loss leads to the constitutive nuclear localization of NF-AT. Second, the Z domain is an in vivo substrate of CKIα, the direct NF-AT kinase identified in this work. Mutation of the CKIα phosphorylation sites within the Z domain also leads to nuclear localization of NF-AT4 in the absence of calcium signaling. The significance of the Z domain as the key element of NF-AT4 nuclear shuttling is underscored by the fact that overexpression of the two kinases which inhibit NF-AT nuclear import, CKIα and MEKK1, fail to suppress the nuclear translocation of the NF-AT4 ΔZ mutant. Similarly, the NF-AT4 ΔZ mutant is nuclear, independent of calcineurin activity, indicating that the loss of the Z domain uncouples NF-AT4 from both inhibitory and stimulatory activities in the cell.

Our analysis of NF-AT4 also uncovered two domains that act to recruit calcineurin and opposing kinases to the proximity of the nuclear location signal mask. The C domain, previously implicated in calcineurin binding, was further defined as a region whose deletion abolishes calcium-stimulated nuclear import of NF-AT4. The serine-rich A domain of NF-AT4 was found to be required for the binding of CKIα, as its loss renders NF-AT4 nuclear import exquisitely responsive to even weak calcineurin activity. The A domain of NF-AT4 mediates CKIα/NF-AT4 interaction and thus promotes the phosphorylation and cytoplasmic localization of NF-AT4. Quite intriguingly, however, was the finding that the nuclear translocation process of the NF-AT4 ΔA mutant was strikingly resistant to MEKK1, the kinase which completely blocks the import of wild-type NF-AT4. We presented here an array of experiments suggesting that MEKK1 inhibits NF-AT4 nuclear import by stabilizing CKIα/NF-AT4 association. The affinity between the A domain and CKIα, and therefore the phosphorylation of NF-AT4, is enhanced by MEKK1 activity. The sum of these findings argues for an intramolecular mechanism regulating the nuclear shuttling of NF-AT4 that is mediated by the opposing actions of phosphatases and kinases (Figure 7F). CKIα directly phosphorylates and binds to the A domain of NF-AT4 and subsequently phosphorylates the Z domain. The phosporylated Z domain then assumes a conformation that blocks the recognition of the NLS sequence by cytoplasmic nuclear translocation factors (Shibasaki etal., 1996; Beals et al., 1997a). Upon calcium influx, activated calcineurin binds the C domain of NF-AT4 and dephosphorylates both the A and Z domains, resulting in the disruption of CKIα NF-AT4 association and exposure of the NLS, respectively (Figure 7F).

CKIα in NF-AT4 Regulation

CKIα belongs to a family of protein serine/threonine kinases that includes at least seven isotypes (Rowles et al., 1991; Graves et al., 1993; Fish et al., 1995; Zhai et al., 1995; Kusuda et al., 1996). Although some members of the family, particularly CKI-ε and -δ, were implicated in DNA repair, this association is largely extrapolated from genetic studies of Hrr25, Hhp1, and Hhp2, the CKI-ε and -δ homologs in yeast (Brockman et al., 1992; Fish et al., 1995; Ho et al., 1997). CKIα lacks a C-terminal domain thought to regulate other CKI isotypes and is generally considered constitutively active (Graves and Roach, 1995; Longenecker et al., 1996). Two enzymatic features of CKIα are consistent with its function as a NF-AT4 kinase. First, the constitutive kinase activity of CKIα makes it an ideal candidate for opposing NF-AT4 nuclear import in the absence of extracellular stimuli. Second, CKIs are unusual in that they prefer prephosphorylated substrates (Flotow et al., 1990). Kinetic studies indicated that CKIs phosphorylate the first S/T residue following an acidic region in the substrate with a high K_m and then extensively phosphorylates neighboring S/T residues with a much lower K_m (Flotow et al., 1990; Flotow and Roach, 1991). Three-dimensional structures of CKIs have revealed anion binding sites that can potentially mediate the interaction with phosphorylated serine and threonine residues in substrates (Xu et al., 1995; Longenecker et al., 1996). Phosphorylation of CKIα substrates, possibly by itself or other kinases, could thus superimpose regulatory features on the otherwise constitutively active CKIα. The association between CKIα and the NFAT4 A domain, which is prephosphorylated by CKIα or other kinases, would bring CKIα into the vicinity of the Z domain and thereby facilitate the phosphorylation of the Z domain by CKIα (Figure 7F).

MEKK1: A Link to Negative Signaling Pathways?

MEKK1 came to light in this study because several human cell lines showed serum-dependent inhibition of NF-AT4 nuclear import. MEKK1 was previously identified as one of the upstream activators of stress signal pathways, where it directly or indirectly participates in the activation of AP1 and NF-κB transcription factors

(Hirano et al., 1996; Atfi et al., 1997; Lee et al., 1997; Read et al., 1997). Interestingly, our analysis showed that activated SEK1 or JNK1 does not inhibit NF-AT4 nuclear import. Additionally, the removal of JNK phosphorylation sites (Chow et al., 1997) on NF-AT4 does not alleviate the inhibition by MEKK1, indicating that MEKK1 must be acting through intermediates other than SEK1 and JNKs. By overexpressing one of the JNK activators, MKK7, it was recently proposed that JNK inhibits NF-AT4 nuclear translocation (Chow et al., 1997). Given that JNK1 or SEK1 activation has no apparent effect on NF-AT4 nuclear import examined here, these data can be somewhat reconciled by proposing that MKK7 is not acting through JNK to suppress NF-AT4 nuclear import but rather through another intermediate, possibly the one activated by MEKK1. In vitro, NF-AT4 is not a good substrate of purified MEKK1 compared to CKIα (data not shown). It is likely, therefore, that MEKK1 activates a distinct kinase, which in turn targets the A domain of NF-AT4. Additional work will be required to determine the identity of the factors downstream of MEKK1 that directly mediate its inhibitory effect on NF-AT4 nuclear import.

In summary, our findings have defined the essential components of the intramolecular NLS masking mechanism controlling NF-AT4 nuclear import. These include the conditionally phosphorylated Z domain on NF-AT4 itself, as well as the kinases CKIα and MEKK1, which maintain and enhance the NLS masking activity of the Z domain. While CKIα functions as a work-horse kinase that maintains NF-AT4 in the cytoplasm of resting cells, the A domain of NF-AT4 provides a platform onto which various signaling pathways can down-regulate NF-AT4 through CKIα.

Experimental Procedures

Protein Purification

Cell extracts were prepared from proliferating HeLa cells. Cells were washed in phosphate-buffered saline (PBS) and lysed in buffer A (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, 1 mM PMSF, and 1 μg/ml each of leupeptin, aprotinin, and pepstatin A). After clearing by centrifugation at 50,000 × g for 60 min, the lysate was fractionated by 40% ammonium sulfate precipitation and dialyzed extensively against buffer A. For the purification of NF-AT4 kinases, GST-NF-AT4(N) affinity chromatography was employed. Bacterially expressed GST-NF-AT4(N) fusion protein was covalently coupled to a solid matrix (Amino-Linker matrix; Pierce). A preclearing column was made by coupling GST to the same matrix. Approximately 800 mg of lysate protein was passed over the GST column three times. The final flow-through was loaded onto the GST-NF-AT4(N) affinity column and washed with lysis buffer. Proteins retained on the column were eluted with 0.5 M NaCl, desalted, further fractionated on a Mono-Q column (Pharmacia), and eluted with a 0.05–1 M NaCl gradient. Fractions were analyzed for NF-AT4 kinase activity and protein composition was visualized by fractionation on SDS-polyacrylmide gels and silver staining. Protein bands were digested with trypsin, and resulting peptides were analyzed by mass spectrometry and sequenced by Edman degradations.

Cell Lines Maintenance and Transfection

HeLa, BHK (baby hamster kidney), and U2OS (human osteosarcoma) cells were maintained in Dulbecco's modified Eagle's media (DMEM) with 10% fetal bovine serum (FBS) supplemented with 2 mM glutamine and 5000 U/ml streptomycin/penicillin. Transfections were done essentially as described (Shibasaki et al., 1996). For calcium ionophore treatment, A23187 (Calbiochem) at a final concentration of 1 μM was used. For metabolic ^{32}P-labeling, transfected BHK cells were incubated with 0.5 mCi ^{32}P-Phosphate (ICN) in phosphate-free DMEM medium for 4 hr. The cells were then lysed in RIPA buffer. NF-AT4 was immunoprecipitated with anti-Myc mAb 9E10.

Plasmid Constructs

Mammalian expression vectors with either Myc, HA, GST, or GFP tags were constructed in pcDNA3 (Invitrogen). The lamin 5′ UTR was placed upstream of the tags to stabilize the transcripts. All cDNA inserts were cloned into these vectors via same sites: a 5′, in-frame XhoI site and an ApaI site at the 3′ end (Shibasaki et al., 1996; Taylor and McKeon, 1997). Mutations were made by PCR methods.

Immunofluorescence Staining

Transfected cells were fixed with 3% formaldehyde in PBS for 10 min and then blocked and permeabilized with 1% milk in PBS/0.1% Triton X-100 for 30 min at room temperature. Primary and secondary antibodies were sequentially incubated with the cells in 1% milk/PBS/0.1% Triton X-100 for 30 min. Coverslips were finally washed with PBS/0.1% Triton X-100, mounted on slides, and examined using a Zeiss Axiophot microscope. The images were captured with a CCD Northern Exposure camera system. For Myc-tagged proteins, the monoclonal 9E10 antibody was used as the primary antibody followed by a goat Cy3-anti-mouse IgG antibody (Zymed). A rabbit polyclonal anti-HA antibody (Santa Cruz) and a FITC-goat anti-rabbit antibody (Zymed) were used for detection of HA-tagged proteins in double staining, or Marina-blue goat anti-rabbit antibody (Molecular Probes) in triple staining. For GST-fused MEKK1 and SEK1 proteins, a monoclonal anti-GST antibody (MBL) was used as the primary antibody and a Cy3- goat anti-mouse antibody as the secondary antibody. To calculate the percentage of cells showing nuclear import or colocalization of NF-AT4 and CKIα, 200 cells from different coverslips were examined.

Immunoprecipitation and Western Blotting

BHK cells were cotransfected with Myc-tagged NFAT4(N) and HA-tagged CKIα(D136N), grown for 24 hr, and lysed in buffer B (20 mM HEPES, 20 mM β-glycerophosphate, 100 mM NaCl, 1 mM EDTA/EGTA, 1 mM DTT, 0.1 mM sodium vanadate, 0.1% Triton X-100, and protease inhibitors) for 20 min on ice. The cell lysates were clarified by centrifugation, fractionated by SDS-polyacrylamide gel electrophoresis, and transferred onto PVDF membranes (Millipore) using a semidry transfer apparatus.

Kinase Assays

To examine the kinase activities of wild-type and mutant CKIα, HA-tagged CKIα, CKIα(K138R), and CKIα(D136N) were immunoprecipitated from transfected BHK cells. Briefly, cells were lysed in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM DTT, 1 mM EGTA, 0.5% Triton X-100, and 0.1 mM PMSF, and the resulting lysates were cleared by centrifugation at 15,000 × g for 10 min. CKIα in the cell lysates was quantified by Western blotting with the anti-HA antibody. Equal amounts of CKIα were immunoprecipitated with the anti-HA antibody and protein G-Sepharose, incubated in 50 μl kinase reaction buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT) containing 0.1 mM ATP, 0.25 μCi [γ-^{32}P]ATP (3000 Ci/mmol), and 1 mg/ml CKI-specific substrate peptide (6031S; NEB). After a 30 min incubation at 25°C, the reactions were terminated by addition of 50 μl 100 mM EDTA and centrifuged to remove protein G-Sepharose. The supernatants were then spotted onto P18 phosphocellulose disks (Whatman) and washed extensively in 75 mM phosphoric acid. Radioactivity retained on the disks was quantified by liquid scintillation. To analyze CKIα phosphorylation of NF-AT4, GST-NF-AT4(N) or 6×His-tagged NFAT4(N)(187-351) fusion proteins were incubated with purified casein kinase I (NEB) in 50 μl of kinase reaction buffer containing 0.1 mM ATP, 0.25 μCi [γ-^{32}P]ATP, and 0.2 mg/ml NF-AT4 fusion protein for indicated periods of time at 25°C. The reaction was then terminated with SDS-sample buffer. Proteins were fractionated on SDS-polyacrylamide gels and phosphate incorporation into GST-NF-AT4(N) fusion proteins quantitated by Phospho-imager (Molecular Dynamics). 6×His-NF-AT4(N)(187-351) was transferred

onto PVDF membrne and digested with trypsin. The resultant peptides were resolved on an HPLC reverse phase C18 column, and selected peptides were sequenced and analyzed by mass spectrometry. Assays for JNK/SEK kinase activities and UV irradiation were performed as previously described (Sanchez et al., 1994).

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